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RESEARCH PAPER

Importance of membrane-bound catechol-O-methyltransferase in L-DOPA metabolism: a pharmacokinetic study in two types of *Comt* gene modified mice

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Background and purpose: Catechol-O-methyltransferase (COMT) metabolizes compounds containing catechol structures and has two forms: soluble (S-COMT) and membrane-bound (MB-COMT). Here we report the generation of a mouse line that expresses MB-COMT but not S-COMT. We compared the effects of deleting S-COMT only or both COMT forms on the pharmacokinetics of oral L-DOPA.
Experimental approach: L-DOPA (10 mg·kg^{-1}) and carbidopa (30 mg·kg^{-1}) were given to mice by gastric tube, and samples were taken at various times. HPLC was used to measure L-DOPA in plasma and tissue samples, and dopamine and its metabolites in brain. Immunohistochemistry and Western blotting were used to characterize the distribution of COMT protein isoforms.
Key results: Lack of S-COMT did not affect the levels of L-DOPA in plasma or peripheral tissues, whereas in the full COMT-knock-out mice, these levels were increased. The levels of 3-O-methyldopa were significantly decreased in the S-COMT-deficient mice. In the brain, L-DOPA levels were not significantly increased, and dopamine was increased only in females. The total COMT activity in the S-COMT-deficient mice was 22–47% of that in the wild-type mice. In peripheral tissues, female mice had lower COMT activity than the males.
Conclusions and implications: In S-COMT-deficient mice, MB-COMT in the liver and the duodenum is able to O-methylate about one-half of exogenous L-DOPA. Sexual dimorphism and activity of the two COMT isoforms seems to be tissue specific and more prominent in peripheral tissues than in the brain.
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Keywords: COMT; S-COMT; MB-COMT; COMT knock-out; pharmacokinetics; L-DOPA; enzyme activity

Abbreviations: 3-OMD, 3-O-methyl DOPA; CMC, carboxymethylcellulose; COMT, catechol-O-methyltransferase; DAB, 3,3'-diaminobenzidine; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; MB-COMT, membrane-bound COMT; PFC, prefrontal cortex; S-COMT, soluble COMT; TBS, Tris-buffered saline

Introduction

Catechol-O-methyltransferase (COMT) is a widely expressed enzyme that O-methylates catecholamines and other compounds carrying a catechol structure, including catechol-oestrogens (Guldberg and Marsden, 1975; Männistö and Kaakkola, 1999). High COMT activity is found in the liver, kidney and gut wall. A single *Comt* gene codes for two separate enzymes, the soluble (S-COMT) and membrane-bound (MB-COMT) forms. S-COMT is more abundant in peripheral tissues while MB-COMT prevails in the brain, particularly in humans (Männistö and Kaakkola, 1999).

The two isoforms of COMT have been proposed to have partially distinct roles: MB-COMT is supposed to be primarily

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involved in the termination of dopaminergic and noradrenergic synaptic neurotransmission at physiologically relevant low concentrations of catecholamines (Roth, 1992). MB-COMT has also been shown to have a higher affinity for catechol substrates and a lower K_m value for dopamine than S-COMT. S-COMT, on the other hand, is a high-capacity enzyme isoform indicated by higher V_{max} values than those of MB-COMT (Lotta *et al.*, 1995). S-COMT is mainly responsible of the elimination of biologically active or toxic, particularly exogenous, catechols and some hydroxylated metabolites. COMT also acts as an enzymatic detoxifying barrier between the blood and other tissues, shielding them from the detrimental effects of hydroxylated xenobiotics (Männistö *et al.*, 1992; Kaakkola *et al.*, 1994; Männistö and Kaakkola, 1999). COMT modulates some excretory functions in the kidney and intestinal tract by modulating the dopaminergic tone (Kaakkola *et al.*, 1994; Eklöf *et al.*, 1997; Hansell *et al.*, 1998). The same is likely to be true in the brain: COMT activity regulates the levels of dopamine, particularly in frontal cortical areas (Yavich *et al.*, 2007), and may therefore be associated with the modulation of several behavioural and cognitive processes (Tunbridge *et al.*, 2006).

Much of our knowledge of COMT has been generated using selective COMT inhibitors that have been introduced to clinical use as adjuncts of the regular L-DOPA/DOPA decarboxylase (DDC) inhibitor therapy of Parkinson's disease (Männistö and Kaakkola, 1999). COMT inhibitors increase peripheral L-DOPA levels and thus improve its partition to the brain by preventing its conversion to 3-O-methyl DOPA (3-OMD) in peripheral tissues. Accordingly, the dose of L-DOPA can be substantially reduced compared with combination therapy without a DDC inhibitor. The dose interval of L-DOPA can also be prolonged. Further, COMT inhibitors should decrease fluctuations of dopamine formation in the brain (Männistö and Kaakkola, 1999).

To further clarify the importance of COMT, mice with genetic deletion of COMT [COMT-knock-out (COMT-KO) mice] have been developed (Gogos *et al.*, 1998). Under normal conditions, these animals do not show any major disturbances in their behaviour or reproduction. We have recently generated a mouse line that does not produce S-COMT by genetically engineering a mutation in one of the two start codons of the *Comt* gene. This is the first report where this line will be partially characterized. This S-COMT-deficient mouse line will lead to a greater understanding of the role of the isoforms. The present study aimed at defining the quantitative role of MB-COMT in the pharmacokinetics and metabolism of orally given L-DOPA. To this end, L-DOPA and 3-OMD levels were measured in the plasma, a number of peripheral tissues and brain regions of the wild-type control mice, the S-COMT-deficient and full COMT-KO mice. Also, immunoreactivity to COMT was characterized in peripheral tissues. Both male and female mice were included in the study to explore sex differences in L-DOPA metabolism and the function of COMT. The results of this study suggest that in the mouse, MB-COMT might have a more important role than previously assumed in the peripheral metabolism of L-DOPA.

Methods

Animals

All animal care and experimental procedures were according to the European Community Guidelines for the use of experimental animals (European Communities Council Directive 86/609/EEC), reviewed by the State Provincial Office of Southern Finland and approved by the Animal Experiment Board in conformance with the current legislation.

Both female and male *Comt* gene-disrupted (COMT-KO mice; age range: 3–6 months), S-COMT-deficient mice (age range: 3–6 months) and the wild-type littermates of both strains were used. Mice were bred in the Laboratory Animal Centre in the University of Helsinki, Finland, breeding heterozygous males and females. To keep the strains viable, they were enriched regularly by mating C57BL/6J females obtained from Harlan, the Netherlands, with heterozygous males. The heterozygous offspring of these couples were used further for breeding. The mice were housed at an ambient temperature of 21–23°C under 12:12 h light cycle with free and continuous access to food pellets and drinking fluid. The phase of the oestrous cycle in female mice was not determined.

Generation of transgenic mouse lines

The *Comt* gene-disrupted mouse strain was originally generated by Gogos *et al.* (1998) on mixed 129/Sv × C57BL/6J background and later backcrossed for more than 20 generations on pure C57BL/6J background.

The S-COMT-deficient strain was constructed by a mutation of the methionine coding start codon (ATG) for S-COMT to threonine (ACC) in the *Comt* gene (Figure 1A). The detailed methodology for producing S-COMT-deficient mice is described in the supplementary material. Briefly, the MET2 sequence (see Figure 1A) was mutated in a plasmid (pLitmus-29, New England Biolabs, MO) that contained a 1.3 kb fragment from the *Comt* gene including exon2, introducing a new restriction site (BshTI). The fragment was cloned back to engineered restriction sites (Bsp1407/Kpn2I) of pGEM11-based vector containing contiguous flanking *Comt* gene regions (2.3 kb upstream and 4.2 kb downstream). This vector contained also Loxed *Neo* and *TK* cassettes within the 4–5 intron and the diptheria toxin A cassette at the 5′ end of the whole *Comt* fragment (Figure 1A). The linearized (XhoI/Mph1103I) plasmid was electroporated in ES cells from which neomycin-resistant clones were selected and treated with *Cre* recombinase. Positive clones were used for blastocyst injection. Chimerical males were mated with C57BL/6J females, and DNA from tail biopsies of F1 pups was typed by PCR (see below). F1 heterozygous mice were mated, and F2 mice of all three genotypes were obtained. Heterozygous and homozygous S-COMT mice are healthy and viable, and they breed normally. This could be expected because the *Comt* gene-disrupted knock-out mice are also normal (Gogos *et al.*, 1998; Haasio *et al.*, 2003).

Genotyping

Genomic DNA was isolated from tail biopsies as described by Laird *et al.* (1991). The *Comt* gene-disrupted mice were geno-

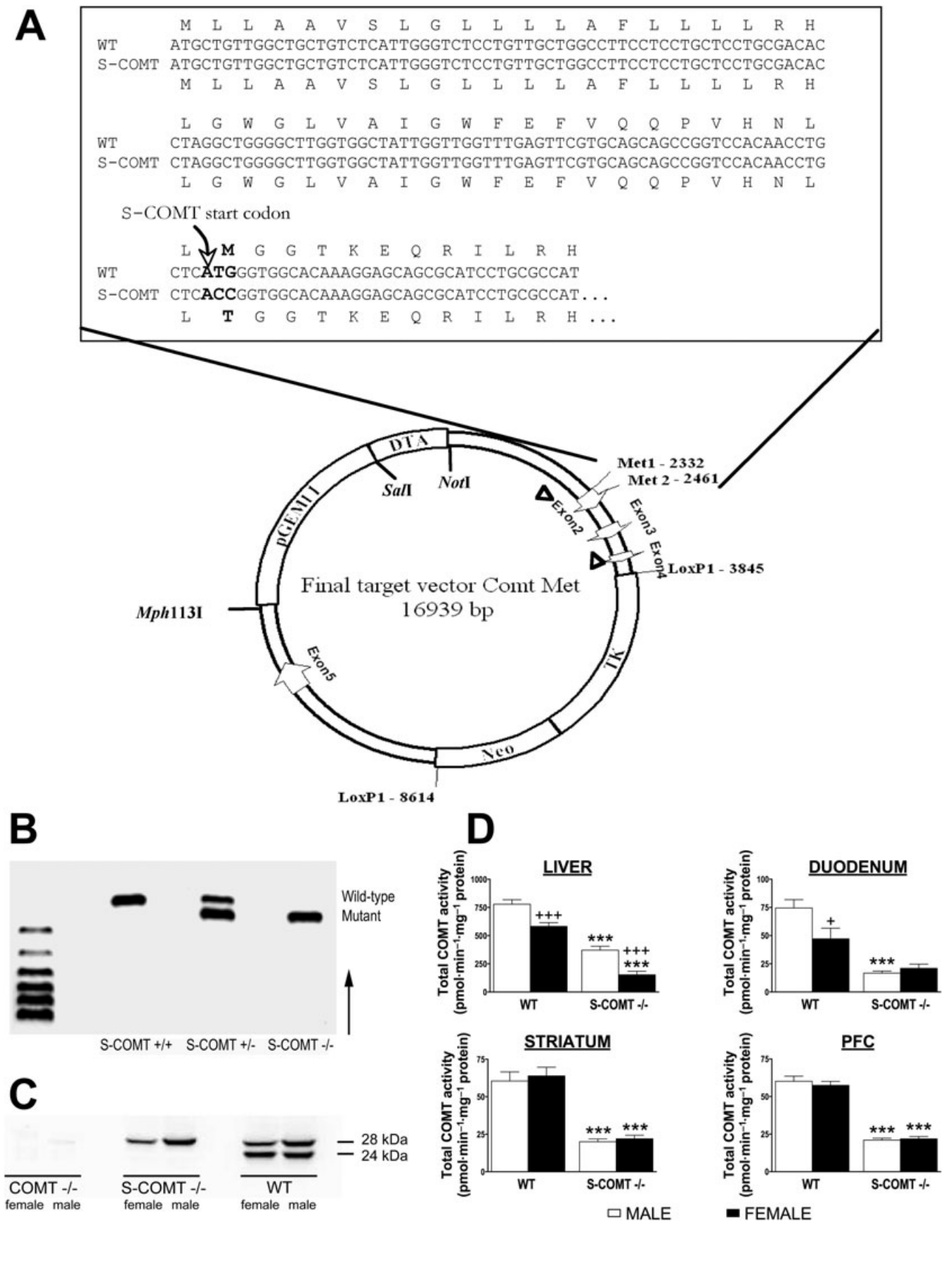
A
M L L A A V S L G L L L L A F L L L L R H
WT ATGCTGTTGGCTGCTGTCTCATTGGGTCTCCTGTTGCTGGCCTTCCTCCTGCTCCTGCGACAC
S-COMT ATGCTGTTGGCTGCTGTCTCATTGGGTCTCCTGTTGCTGGCCTTCCTCCTGCTCCTGCGACAC
M L L A A V S L G L L L L A F L L L L R H
L G W G L V A I G W F E F V Q Q P V H N L
WT CTAGGCTGGGGCTTGGTGGCTATTGGTTGGTTTGAGTTCGTGCAGCAGCCGGTCCACAACCTG
S-COMT CTAGGCTGGGGCTTGGTGGCTATTGGTTGGTTTGAGTTCGTGCAGCAGCCGGTCCACAACCTG
L G W G L V A I G W F E F V Q Q P V H N L
S-COMT start codon
L M G G T K E Q R I L R H
WT CTCATGGGTGGCACAAAGGAGCAGCGCATCCTGCGCCAT
S-COMT CTCACCGGTGGCACAAAGGAGCAGCGCATCCTGCGCCAT...
L T G G T K E Q R I L R H ...
DTA
SalI
NotI
pGEM11
Met1 - 2332
Met 2 - 2461
Exon2
Exon3
Exon4
LoxP1 - 3845
Final target vector Comt Met
16939 bp
Mph1131
Exon5
TK
Neo
LoxP1 - 8614
B
Wild-type
Mutant
S-COMT +/+
S-COMT +/-
S-COMT -/-
C
28 kDa
24 kDa
COMT -/-
female male
S-COMT -/-
female male
WT
female male
D
LIVER
DUODENUM
STRIATUM
PFC
Total COMT activity (pmol·min⁻¹·mg⁻¹ protein)
WT
S-COMT -/-
+++
***
MALE
FEMALE

Figure 1 (A) Constructed ~17 kb targeting vector containing ~2.5 kb long 5′ homology arm up to Mutated MET2, from Mut-MET2 to floxTK/NEO cassete ~1.4 kb central arm, ~4.7 kb floxTKNeo cassette and ~4 kb short 3′ homology arm. Positions of the *Comt* exons, vector and selection cassettes are indicated as well as the restriction sites used to linearize and the LoxP1 sites flanking the Neo and TK cassettes removed after recombination in ES cells. Greater detail of the sequence around Met2 in Exon 2 is shown. The DNA sequence of soluble catechol-O-methyltransferase (S-COMT)-deficient mutant is shown, and the sequence of the corresponding protein product is aligned on top (wild type) and bottom (S-COMT). The bases mutated are shown in bold font. A new *AgeI* restriction site (ACCGGT) for genotyping was introduced along with the Met44Thr mutation. (B) PCR genotyping of S-COMT-deficient animals showing wild-type (S-COMT +/+), heterozygous (S-COMT +/–) and homozygous (S-COMT –/–) genotypes consisting of wild-type allele, mutated allele or both alleles. (C) Western blot analysis visualizing the long (28 kDa) and short (24 kDa) catechol-O-methyltransferase (COMT) transcripts in the liver. (D) Total COMT activities in the liver, duodenum, striatum and prefrontal cortex (PFC) of wild-type (WT) and S-COMT-deficient (S-COMT –/–) mice. $^{+}P < 0.05$ and $^{+++}P < 0.001$ differs significantly from corresponding male. $^{***}P < 0.001$ differs significantly from corresponding wild-type mice. *n* (male/female) = 12/12 (WT) and 6/6 (S-COMT –/–).

typed as reported in Tammimäki *et al.* (2008). The details are given in the supplementary material.

The amplified fragments were visualized by ethidium bromide staining under ultraviolet light after electrophoresis in a 2% agarose gel (Figure 1B).

Western immunoblotting

For Western immunoblotting, the liver samples were collected and rinsed in physiological saline solution. Immediately after dissection the tissues were placed in ice-cold centrifuge tubes on dry ice to minimize decomposition. All the tissue samples were frozen and stored in –80°C until analysed. The details are given in the supplementary material.

Assay of COMT activity

Total COMT activity was measured from duodenal and liver samples and from two brain areas [striatum and medial prefrontal cortex (PFC)]. After decapitation of the mice, the brain was rapidly excised, rinsed with ice-cold physiological saline solution and placed on an ice-cooled coronal mouse brain matrix (Stoelting, Wood Dale, IL). A 2-mm coronal slice was cut off with two razor blades, and the dorsal striatum was punched out below the corpus callosum from this slice by a sample corer (i.d. 2 mm) with a plunger (Fine Science Tools GmbH, Heidelberg, Germany). A second 2 mm coronal slice was cut off with two razor blades, and the PFC was cut off with a surgical knife. The brain tissues were immediately placed in frozen centrifuge tubes to be cooled on dry ice. Peripheral tissues were rapidly dissected and placed in pre-cooled plastic centrifuge tubes on dry ice as described earlier in the Western immunoblotting section. Samples were stored in –80°C until assayed. The COMT assay was performed with slight modifications from methods described earlier (Nissinen and Männistö, 1984; Reenilä *et al.*, 1995). Further details of the assay are reported in the supplementary material.

The protein concentration of the samples was determined with Pierce protein assay kit based on bicinchoninic acid method (Pierce, Rockford, IL). Specific activity of COMT is expressed as pmoles vanillic and isovanillic acid formed per minute per milligram of protein in the sample.

COMT immunohistochemistry

Animals and tissue sampling. For tissue sampling of the paraffin-embedded tissues, animals were deeply anaesthetized with chloral hydrate (450 mg·kg^{-1} i.p.) and then perfused with saline and thereafter with 4% paraformaldehyde solution. After perfusion, the internal organs were removed and placed in 10% paraformaldehyde solution until paraffin embedding and sectioning into 4 mm paraffin-embedded sections using a sliding microtome (Leica SM2000R, Leica Microsystems Inc., Wetzlar, Germany).

Light microscopic immunohistochemistry. For paraffin-embedded mouse tissues, immunohistochemistry was performed using a slightly modified version of the protocol described in Myöhänen *et al.* (2008). The details are given in the supplementary material.

Pharmacokinetic studies and sample collection

The mice were given L-DOPA (10 mg·kg^{-1}) and the DDC inhibitor carbidopa (30 mg·kg^{-1}) suspended in 0.25% carboxymethylcellulose (CMC) gel with a plastic gastric tube in a volume of 5 mL·kg^{-1} of body weight. From each mouse, two blood samples were collected in Microvette capillary blood collection tubes (Sarstedt, Nümbrecht, Germany) containing EDTA. The first sample (100 mL) was taken from the saphenous vein and the second, terminal sample (300 mL) from the jugular vein. The two samples from each individual mouse were taken at random time points, and we treated enough mice to get at least six independent samples of each genotype at every time point (0, 30, 60, 90 and 120 min). The blood samples were centrifuged at 4900× *g* at 4°C for 10 min and the plasma was frozen and stored in –80°C. After the terminal blood sample, the mice were killed by decapitation and the tissues were dissected as described earlier in the Western immunoblotting and COMT activity-assay sections.

Assay of monoamines in plasma and tissue samples

Plasma samples. 10 mL of the plasma sample was added to 90 mL of 0.2 M $HClO_4$ to sediment the proteins. The sample was centrifuged for 35 min at 20800× *g* at 4°C. The supernatant was transferred to 0.5 mL Vivaspin filter concentrators (10,00 MWCO PES; Vivascience AG, Hannover, Germany) and centrifuged additionally for 35 min at 8600× *g* at 4°C. The filtrate was then analysed using HPLC with electrochemical detection. Details are given in the supplementary material.

Tissue samples. The tissues were prepared as reported previously (Airavaara *et al.*, 2006) and then analysed for monoamines and their metabolites as for the plasma samples.

Vascular space contribution. As the animals were not perfused, the contribution of blood L-DOPA and 3-OMD levels was estimated when judging the tissue concentrations. In case of blood dopamine and its metabolites, blood contribution was negligible. Details of this procedure are given in the supplementary material. We did not recalculate the tissue levels of L-DOPA and 3-OMD. The contribution of the blood to total tissue concentrations was always less than 10% and evidently very similar in all animals. Therefore, comparisons of the three groups were not invalidated.

Statistics

The results are shown as mean ± SEM. Concentration-time curve was calculated from group means as only two blood samples and one tissue sample were collected from each animal. The L-DOPA, dopamine, and metabolite levels and COMT activities were compared with one-way analysis of variance (ANOVA) followed by Scheffe's test. The effect of sex and genotype was calculated using two-way ANOVA followed by Scheffe's test in cases where significant F-values were found. Area under the concentration-time curve ($AUC_{0–120\ min}$) was calculated according to the trapezoidal rule. SAS Institute Statview 5.0 software was used in all calculations.

Materials

L-DOPA, 3-OMD, dopamine, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were from Sigma Chemical Co., (St. Louis, MO), and carbidopa was from Orion Pharma (Espoo, Finland). CMC was from Fluka Chemie (Steinheim, the Netherlands) and chloral hydrate from Merck KgaA (Darmstadt, Germany).

The drug target/receptor nomenclature in this paper follows Alexander *et al.* (2008).

Results

Generation and characterization of S-COMT-deficient mice

Genetically engineered S-COMT-deficient mice carry a point mutation in *Comt* gene start codon 2 (corresponding to 'ATG' position +30 in exon2 of *Comt* gene; see Ulmanen and Lundström, 1991), which disables the translation of soluble COMT. The mice were produced and maintained on a C57BL6/J background. Heterozygous and homozygous S-COMT mice were healthy and viable, and they bred normally.

Western immunoblotting of the liver COMT

Western blot analysis of total liver extracts using a polyclonal COMT antibody revealed that the relative intensity of the longer MB-COMT (28 kDa) and the shorter S-COMT (24 kDa) bands was equal in wild-type male mice, while in females, the expression of MB-COMT was slightly weaker than males. As expected, S-COMT-deficient animals expressed only MB-COMT. Similarly to wild-type mice, the relative abundance of MB-COMT was lower in females than in males. No COMT protein was detected in COMT-KO animals (Figure 1C).

COMT activity in peripheral and brain tissues

In both the wild-type and S-COMT-deficient mice, total COMT activity in the liver was lower in female than in male mice ($P < 0.001$) (Figure 1D). In duodenal samples, sex-related differences were found only in wild-type animals ($P < 0.05$). Lack of the S-COMT isoform resulted in a clear reduction of total COMT activity in the liver of both sexes [by 74% in females ($P < 0.001$), by 53% in males ($P < 0.001$)], whereas in the duodenum, significant reduction was seen only in males (by 78%, $P < 0.001$). In the striatum and the PFC, a reduction of total COMT activity in S-COMT-deficient animals was also observed ($P < 0.001$), but no sex-related differences were found. COMT-KO animals had no detectable COMT activity in any tissue or region studied.

COMT protein distribution by immunohistochemistry

In the wild-type mice, COMT was abundantly expressed in the duodenal epithelial cells and microvilli (Figure 2A). Some COMT immunoreactivity was detected in the duodenum of the S-COMT-deficient mice (Figure 2B), but the intensity of COMT immunostaining was noticeably lower than that in the wild-type animals. As expected, no COMT immunoreactivity was detected in the duodenum of the COMT-KO mice (Figure 2C). Similar results were obtained in the liver, where we found high levels of expression of COMT in various types of liver cells in the wild-type mice (Figure 2D), significantly less COMT staining in the S-COMT-deficient mice (Figure 2E) and lack of any immunoreactivity in the COMT-KO mice (Figure 2F). It should be noted that both in the duodenum and the liver of the S-COMT-deficient animals, MB-COMT was seen in intracellular membranes.

Effect of COMT isoforms on absorption and metabolism of L-DOPA after oral administration of L-DOPA and carbidopa

Plasma L-DOPA levels were elevated after a single dose of L-DOPA in both sexes and all genotypes, reaching peak values at 30 min (wild-type and S-COMT-deficient mice) or 60 min (COMT-KO mice) (Figure 3A). Furthermore, the increase in apparent L-DOPA C_{max} levels was higher in COMT-KO animals than in S-COMT-deficient mice (~55% vs. ~30% compared with their wild-type littermates). In the male mice, the absence of COMT prolonged the elimination of L-DOPA, but the same was not seen in the female mice. Otherwise, there was no sex-related difference in the overall L-DOPA pharmacokinetics. The $AUC_{0–120\ min}$ values confirmed that L-DOPA levels were significantly increased only in the COMT-KO mice of both sexes ($P < 0.001$ in males, $P < 0.01$ in females).

Both duodenal and hepatic L-DOPA levels (Figure 3B,C) followed closely the corresponding levels in the plasma, but some striking sex-related differences were observed. In the liver, the absence of both COMT isoforms increased $AUC_{0–120\ min}$ values of L-DOPA in both sexes ($P < 0.001$ in males, $P < 0.01$ in females) compared with wild-type mice. In the duodenum, significant elevation was observed only in male COMT-KO mice ($P < 0.001$). L-DOPA levels were very low and no genotype-related differences in peripheral L-DOPA levels were seen under basal conditions (Figure 3).

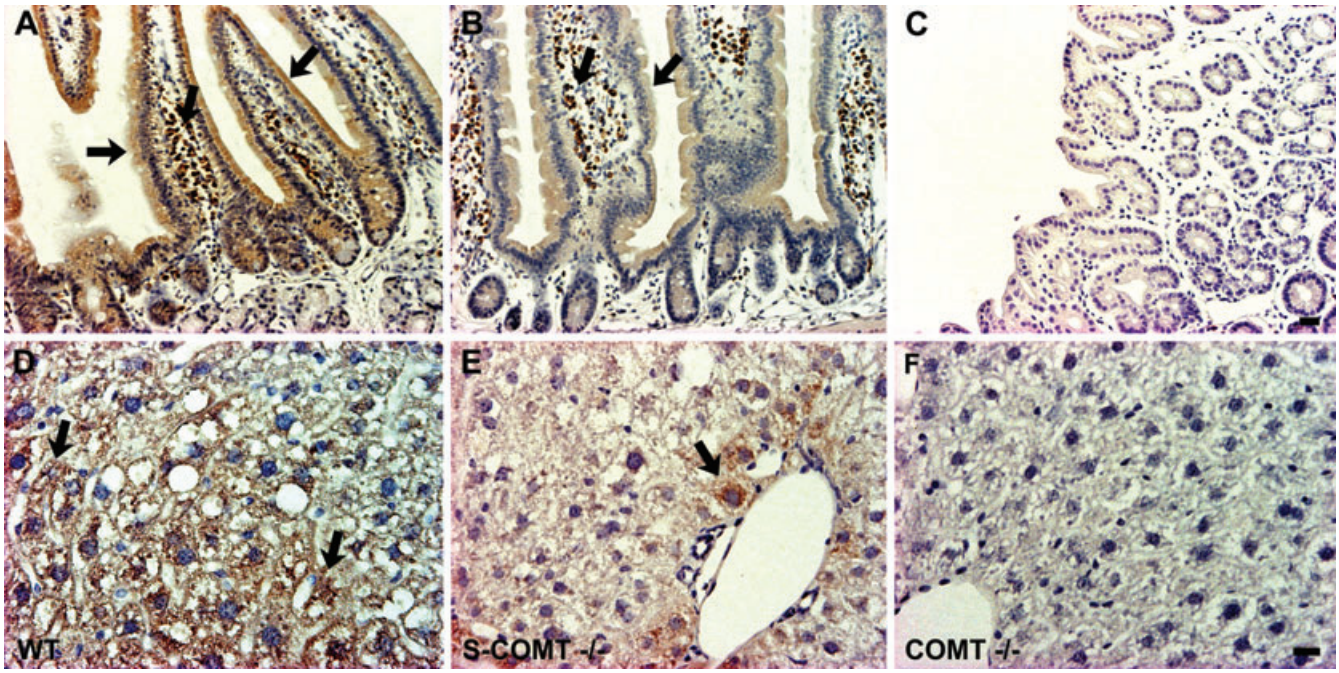

Figure 2 Light-microscopic photomicrographs presenting the distribution of COMT in the duodenal (A–C) and liver (D–F) tissue of wild-type (WT), soluble catechol-O-methyltransferase (S-COMT)-deficient (S-COMT –/–) and catechol-O-methyltransferase (COMT) knock-out (COMT –/–) mice. COMT was visualized with DAB (brown colour) and nuclei were counterstained with Mayer's haematoxylin (blue colour). The lack of S-COMT clearly reduced the intensity of COMT-immunostaining. Moreover, in the S-COMT-deficient liver cells (E), COMT is seen more in the intracellular membranes than the plasma membrane. Scale bars are 20 μm (A–C) and 30 μm (D–F).

Effect of COMT isoforms on 3-OMD levels in peripheral tissues after oral administration of L-DOPA and carbidopa

Consistent with COMT activity measurements, plasma 3-OMD levels were significantly reduced in both sexes as a function of decreasing COMT. No 3-OMD was found in the COMT-KO mice. In wild-type mice, the levels of 3-OMD and the respective $AUC_{0–120\ min}$ values were lower in females than in males ($P < 0.001$) (Figure 4A). 3-OMD levels in tissues were similar to those determined in the plasma for both sexes (Figure 4B,C).

Effect of COMT genotype on L-DOPA, 3-OMD, dopamine, DOPAC and HVA levels in the brain after oral administration of L-DOPA and carbidopa

We did not observe any sex or genotype effects in the $AUC_{0–120\ min}$ values of L-DOPA levels in the striatum and the PFC (Figure 5A). The elevation of L-DOPA was not as pronounced as in the peripheral tissues. Also, the apparent C_{max} of L-DOPA was generally observed later in the striatum and the PFC than in the plasma (Figure S1, supplementary material). Central 3-OMD levels were strikingly similar to the levels in plasma and peripheral tissues, with the male wild-type mice having the highest 3-OMD concentrations (Figure 5B).

In striatal and PFC dopamine levels, no genotype effects were seen under basal conditions (Figure S1C, supplementary material). As for basal dopamine metabolite levels, significant increases of DOPAC (Figure S1D, supplementary material) were seen only in the PFC of the COMT-KO mice in which HVA was absent in both brain areas (Figure S1E, supplementary material). Lack of S-COMT did not significantly modify basal DOPAC or HVA levels in either brain area (Figure S1D,E, supplementary material).

After the administration of L-DOPA, female COMT-KO mice had elevated $AUC_{0–120\ min}$ values of dopamine in both the striatum ($P < 0.05$) and the PFC ($P < 0.01$) compared with the values in the corresponding wild-type mice, whereas in males the dopamine levels were unaffected by the genotype (Figure 5C). In the striatum, S-COMT deficiency increased the elevation of DOPAC $AUC_{0–120\ min}$ values only in the female mice (by 77%, $P < 0.01$) compared with the wild-type mice (Figure 5D), while the reduction of HVA $AUC_{0–120\ min}$ values was seen in both sexes ($P < 0.001$) of S-COMT-deficient animals (Figure 5E). In the PFC, there was an elevation of DOPAC $AUC_{0–120\ min}$ values in both sexes of S-COMT-deficient mice (95% in males, $P < 0.01$, and 153% in females, $P < 0.001$) compared with the levels in the corresponding wild-type mice, while the reduction of HVA $AUC_{0–120\ min}$ values was seen only in the male mice ($P < 0.05$). In the COMT-KO mice, the elevation of DOPAC $AUC_{0–120\ min}$ values was observed in both sexes, the levels being highest in the PFC of the male mice (281% elevation compared with wild-type mice, $P < 0.001$). No HVA was detected in the COMT-KO animals.

Discussion

This is the first report of S-COMT-deficient animals and their use to dissect the role of the two COMT forms in L-DOPA

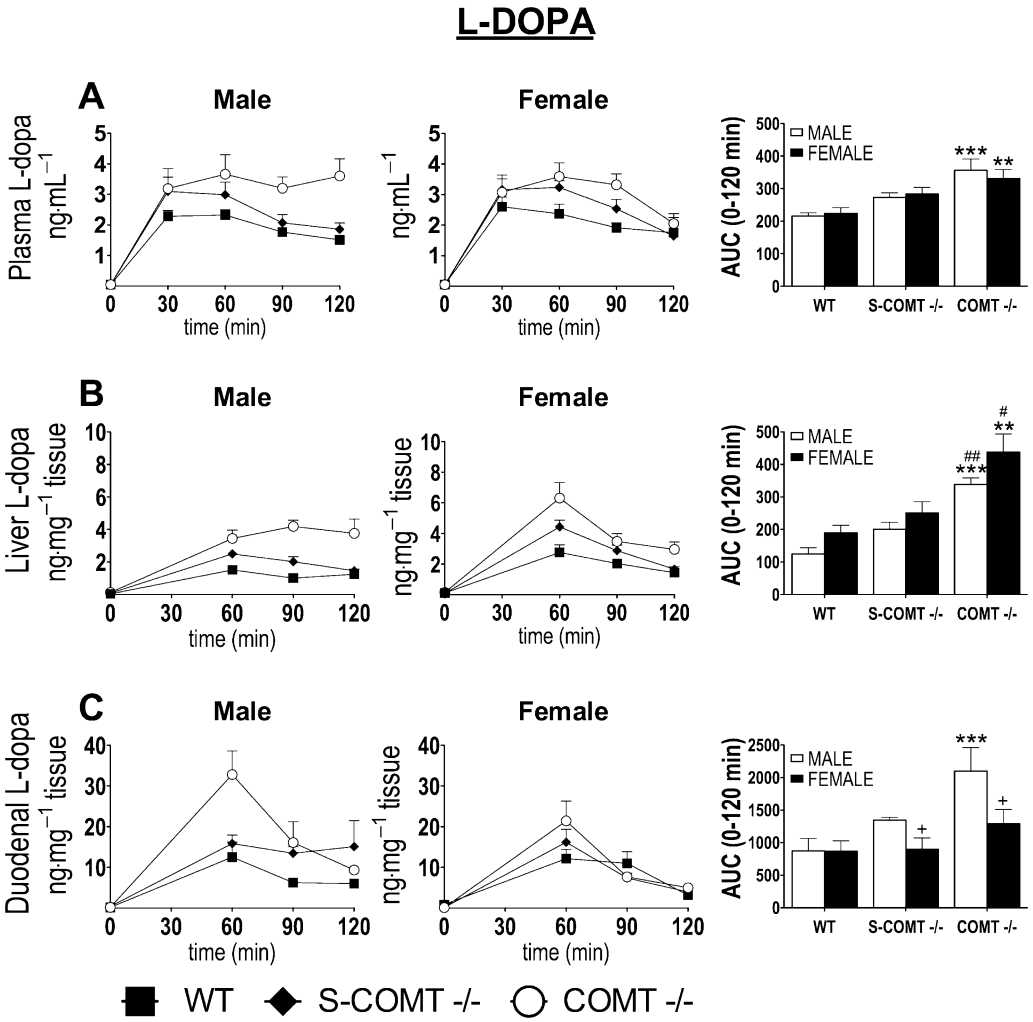

Figure 3 Effect of catechol-O-methyltransferase (COMT) genotype [soluble catechol-O-methyltransferase (S-COMT) deficiency or COMT knock-out] on the time course and $AUC_{0-120\ min}$ of the plasma (A), liver (B) and duodenal (C) L-DOPA concentrations after oral administration of L-DOPA and carbidopa (10 mg·kg^{-1} and 30 mg·kg^{-1} respectively). The data represent group means ± SEM. $^{+}P < 0.05$ differs significantly from corresponding male. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ differs significantly from corresponding S-COMT deficient mice. $^{**}P < 0.01$ and $^{***}P < 0.001$ differs significantly from corresponding wild-type (WT) mice. *n* (male/female) = 13/13 (WT), 6/7 (S-COMT –/–) and 6/7 (COMT –/–).

pharmacokinetics. Our results suggest a much more important role for MB-COMT in the mouse peripheral tissues than has previously been anticipated (Ellingson *et al.*, 1999; Männistö and Kaakkola, 1999).

In previous Western blot analyses in rodents, S-COMT was at least three times more abundant than MB-COMT in the majority of rat tissues (Tenhunen *et al.*, 1994) and at least twice as abundant than MB-COMT in the hypothalamus and liver of mice (Hill *et al.*, 2007). In our study, both forms of COMT seemed to be equally abundant in the male mouse liver, whereas in the female liver, more S-COMT was present. MB-COMT was seen in the intracellular membranes of the immunostained S-COMT-deficient animals, supporting the earlier cell culture results (Ulmanen *et al.*, 1997).

Our COMT activity assay revealed that the total COMT activity in the liver and the duodenum was lower in the wild-type female than male mice. The apparent gender-related difference in total COMT activity has been seen also in the human liver (De Santi *et al.*, 1998). MB-COMT activity in the S-COMT-deficient mice was sex dependent only in the liver. Our results support only partially the previous findings of Ellingson *et al.* (1999) in regard to relative activities of S-COMT and MB-COMT. They concluded that the activity of S-COMT was approximately 30-fold to that of MB-COMT in the liver, whereas in our study the relative contribution of S-COMT was much less. Although an increase of MB-COMT in the liver could be compensatory to the absence of S-COMT, our Western blot results provide no evidence of up-regulation. We suggest that S-COMT accounts for ~50% of the total COMT activity in the male liver and ~70% in the female liver. Two points may explain these differences. A quantitative biochemical separation of the COMT forms is challenging and prone to contamination, easily leading to overestimation of S-COMT as previously recognized (Ellingson *et al.*, 1999). Also, species- and strain-related variations in COMT activity may occur (Männistö and Kaakkola, 1999; Grice *et al.*, 2007).

In the duodenum, the larger relative reduction in total COMT activity (by 78% in the male vs. by 55% in the female)

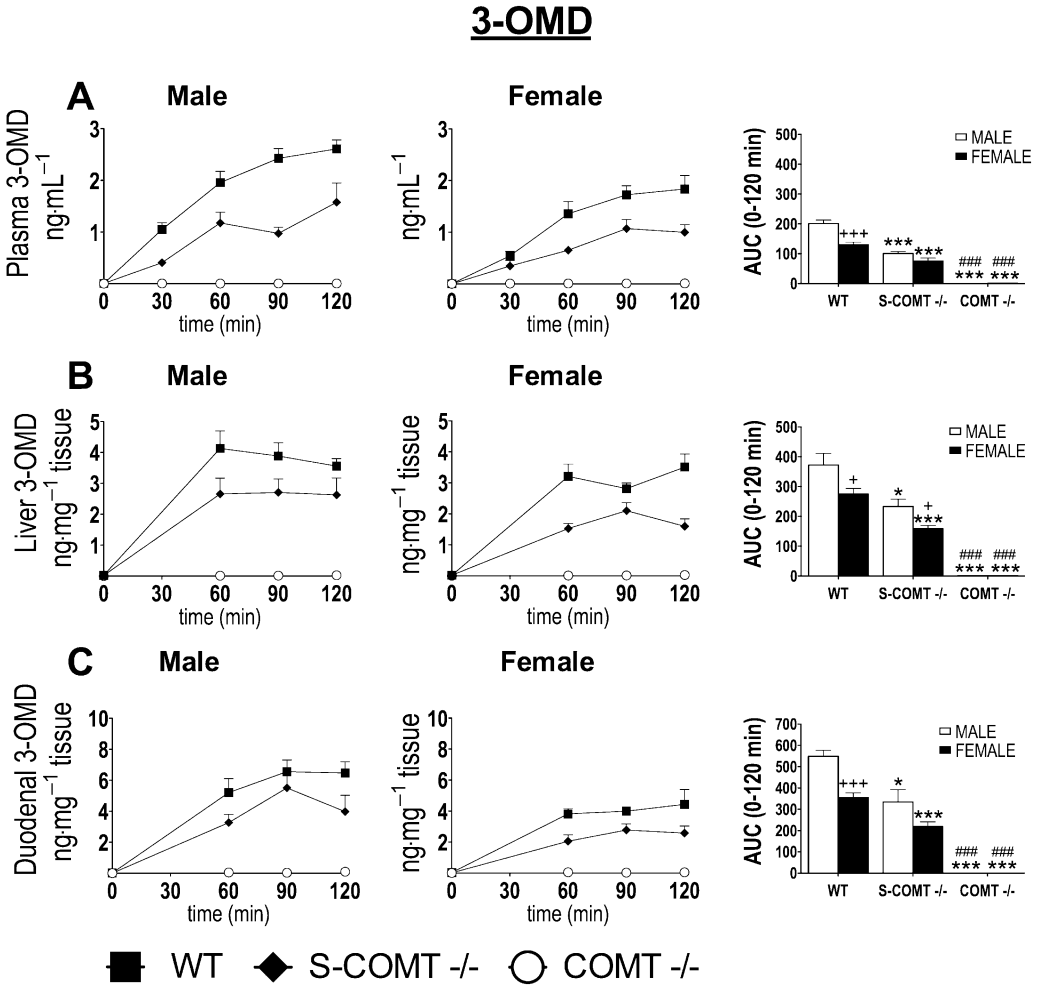

Figure 4 The effect of catechol-O-methyltransferase (COMT) genotype [soluble catechol-O-methyltransferase (S-COMT) deficiency or COMT knock-out] on the time course and $AUC_{0–120\ min}$ of the plasma (A), liver (B) and duodenal (C) 3-O-methyl DOPA (3-OMD) levels after oral administration of L-DOPA and carbidopa (10 mg·kg^{-1} and 30 mg·kg^{-1} respectively). The data represent group means ± SEM. $^{+}P < 0.05$ and $^{+++}P < 0.001$ differs significantly from corresponding male. $^{\#\#\#}P < 0.001$ differs significantly from corresponding S-COMT-deficient mice. $^{*}P < 0.05$ and $^{***}P < 0.001$ differ significantly from corresponding wild-type (WT) mice. *n* (male/female) = 13/13 (WT), 6/7 (S-COMT –/–) and 6/7 (COMT –/–).

suggests, in contrast to the liver, an accentuated role for S-COMT in the male mice. In the striatum and the PFC, S-COMT deficiency accounted for 60–70% reduction of total COMT activity. In concurrence with previous studies (Huotari *et al.*, 2002a), no sex-related differences were seen in total COMT activity in the brain tissues studied.

Recent findings by Øverbye and Seglen (2009) may partially explain some of these inconsistencies. Additional variants of COMT, derived by post-translational modifications such as phosphorylation, have been identified. As phosphorylated and non-phosphorylated variants of both S-COMT and MB-COMT were found both in soluble and sedimentable fractions, it was suggested that the COMT variants could be better classified by size than by membrane association. Unfortunately, it is not yet known how phosphorylation affects COMT activity. Also, a new *Comt-like* gene, COMT2, has recently been identified by Du *et al.* (2008). This gene is expressed in the outer and inner hair cell of the cochlea as well as in vestibular hair cells and is essential for auditory and vestibular function. The authors suggest that COMT2 may also be expressed elsewhere in the brain and that it would be up-regulated to compensate for an inactive COMT gene. However, several highly sensitive analyses of COMT-KO mice (Huotari *et al.*, 2002a,b; 2004; Tammimäki *et al.*, 2008) have not shown any HVA formation in these mice, ruling out another, generally available COMT-like activity, compensating for the lack of COMT.

In our pharmacokinetic studies, the L-DOPA levels in the plasma and the liver of COMT-KO mice were double of those in the wild-type mice. Visual inspection of the concentration-time curve showed that in male mice, the elimination time of plasma L-DOPA was increased. In females, the levels of the L-DOPA metabolite, 3-OMD, in the plasma were lower than in males, which is in accordance with the lower total COMT activity in the duodenum and the liver. Also in the liver, L-DOPA levels were higher and 3-OMD levels lower in the female than male mice. In the duodenum, a significant genotype-dependent increase in L-DOPA levels was found only in the male COMT-KO mice. Undoubtedly, the importance of COMT in the peripheral metabolism of L-DOPA is

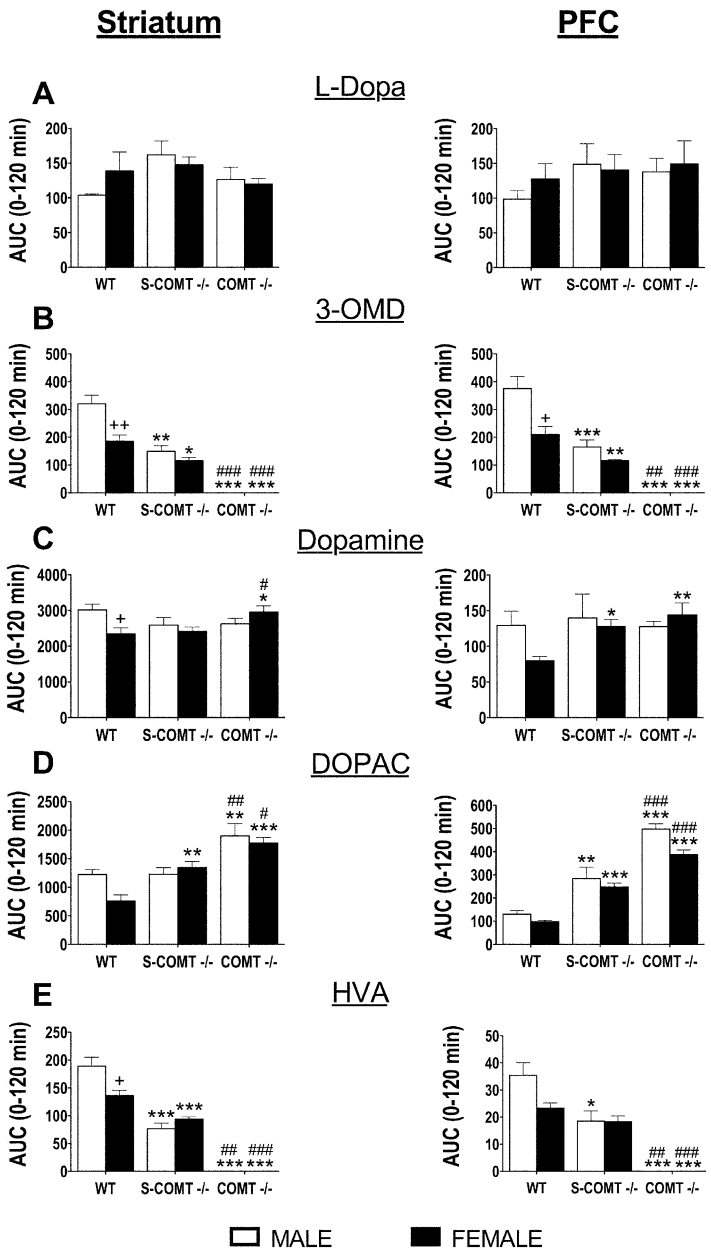

Figure 5 The effect of genotype [soluble catechol-O-methyltransferase (S-COMT) deficiency or catechol-O-methyltransferase (COMT) knock-out] on the $AUC_{0-120\ min}$ of striatal and prefrontal cortex (PFC) levels of L-DOPA (A), 3-O-methyl DOPA (3-OMD) (B), dopamine (C), dihydroxyphenylacetic acid (DOPAC) (D) and HVA (E) after oral administration of L-DOPA and carbidopa (10 mg·kg^{-1} and 30 mg·kg^{-1} respectively). The data represent group means ± SEM. $^{+}P < 0.05$ and $^{++}P < 0.01$ differ significantly from corresponding male. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ differ significantly from corresponding S-COMT-deficient mice. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ differ significantly from corresponding wild-type (WT) mice. *n* (male/female) = 13/13 (WT), 6/7 (S-COMT −/−) and 6/7 (COMT −/−).

greater in male than female mice. The sex-dependent impact of the S-COMT isoform is less clear.

L-DOPA levels in the PFC or the striatum were not genotype- or sex-dependent, although a slight trend towards elevated levels of L-DOPA were seen in the PFC of COMT-KO mice. The 3-OMD levels in both brain areas were much lower in the female than in the male mice. In addition, only the female COMT-KO mice had slightly elevated striatal and PFC dopamine levels compared with the corresponding wild-type animals. 3-OMD is readily transported through the blood–brain barrier and, thus, the brain 3-OMD levels reflect the levels in plasma and peripheral tissues. These results on 3-OMD and L-DOPA levels were consistent with earlier results by Huotari *et al*. (2002a), who failed to show a significant increase in L-DOPA levels in the brain tissue of COMT-KO animals. Also, in this earlier study, elevated dopamine levels after L-DOPA administration were found only in the PFC of both genders, whereas in the present study, elevation occurred both in the striatum and the PFC, but only in female mice. The gender differences may be due to the various effects of oestrogen on dopamine turnover as explained below. Also, the different dosing regimens may have an effect on the

divergent results from these two studies: the bioavailability of oral L-DOPA in our study may have been much lower than in the previous one where L-DOPA was given intraperitoneally (Huotari *et al*., 2002a).

Sex differences in COMT-KO mice have previously been reported in terms of locomotor effects after administration of amphetamine (Huotari *et al*., 2004), GBR12909-induced increase in dopamine levels in the striatum (Huotari *et al*., 2002b), ethanol consumption (Tammimäki *et al*., 2008) and compensatory expression levels of liver proteins (Tenorio-Laranga *et al*., 2009). These apparent sex-related differences might be explained by an oestrogen-induced, time- and dose-dependent down-regulation of COMT (Xie *et al*., 1999). However, Hill *et al*. (2007) reported unexpectedly that oestrogen up-regulates the expression of COMT in the hypothalamus of male mice but not in the liver and the PFC. It appears that the regulatory action of oestrogen may be tissue specific. These actions of oestrogens and the variability of the expression and distribution of the two different oestrogen receptors (ERα and ERβ) (Albertazzi and Purdie, 2001; Kuhl, 2005) could be one factor contributing to the discrepant responses seen in our study. Sexually dimorphic and tissue-specific actions of oestrogen have also been found to affect dopamine turnover through modulation of the dopamine transporter (McEwen and Alves, 1999). Collectively, these results indicate a more complex, still undefined role for oestrogens in modulating dopamine metabolism through a variety of proteins.

The present results point out some other interesting issues regarding a COMT inhibitor treatment in humans. First, peripheral, particularly hepatic, COMT seems to have a more important role in the metabolism of L-DOPA than the brain COMT. Although L-DOPA levels were clearly elevated in plasma and peripheral tissues of the COMT-KO mice, the striatal and PFC levels were similar in all genotypes. Therefore, COMT inhibitors that can penetrate into the brain may not offer a significant advantage over those confined to the periphery. This has been suggested also earlier based on COMT inhibitor studies (Männistö and Kaakkola, 1999). Second, our study suggests that females might respond differently from males to a COMT inhibitor treatment. However, to the best of our knowledge, the clinical reports have not shown any sex difference in either the therapeutic or the adverse effects of COMT inhibitors. Third, the only minor dopamine elevation in the striatum in the full COMT-KO mice does not invalidate the importance of COMT inhibitors as adjuncts in the L-DOPA treatment of Parkinson's disease. The major effect of peripheral COMT inhibition is to prolong and smooth out the kinetics of L-DOPA entry into the brain. The levels of striatal dopamine are not necessarily elevated, but they are more sustained than without COMT inhibition.

In conclusion, the present results indicate that MB-COMT has a more important role in peripheral L-DOPA metabolism in the mouse than has been previously assumed. Although low oral doses of L-DOPA used in this study may result in an overestimation of the importance of MB-COMT, our results clearly demonstrate that MB-COMT can effectively compensate for the absence of S-COMT. We also found that the relative importance of the two COMT isoforms may be sex and tissue dependent.

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Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Text S1 Generation of the mouse line that does not express S-COMT
Text S2 Western immunoblotting
Text S3 Light microscopic immunohistochemistry
Text S4 COMT activity-assay
Text S5 Assay of monoamines in plasma and tissue samples
Text S6 Vascular space contribution

Figure S1 The effect of COMT genotype (S-COMT deficiency or full COMT-ko) on the time course of striatal and PFC levels of L-dopa (A), 3-OMD (B), dopamine (C), DOPAC (D) and HVA (E) after oral administration of L-dopa and carbidopa (10 $mg \cdot kg^{-1}$ and 30 $mg \cdot kg^{-1}$, respectively). Data represents group means. *n* (male/female) = 13/13 (WT), 6/7 (S-COMT –/–), 6/7 (COMT –/–).

Table S1 Primers used for COMT_Met2 Target vector construction and testing.

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